Exhibit 7

Essential role of CD91 in re-presentation of gp96-chaperoned peptides

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Heat shock proteins (HSPs) such as gp96 are released from cells as a result of necrotic cell death. The ability of endogenous HSP–peptide complexes to elicit antigen-specific T cells requires re-presentation of the chaperoned peptides by antigen-presenting cells. Re-presentation requires the uptake of HSP–peptide complexes through a receptor, suggested to be the low-density lipoprotein receptor-related protein or CD91. We have used short interfering RNA for CD91 to show that, as antigen-presenting cells lose expression of CD91, their re-presenting ability undergoes a corresponding and dramatic decline. Furthermore, as the cells recover from extinction of CD91 expression, they regain the ability to re-present peptides. The ability of cells to bind α_2 macroglobulin, a previously known CD91 ligand, or HSP gp96, and their ability to process peptides chaperoned by α_2 macroglobulin, undergo identical variations. These results have been obtained from studies *in vitro* and from an assay that measures re-presentation *in vivo*. In additional studies *in vivo*, protective tumor immunity elicited by tumor-derived gp96–peptide complexes is shown to be abrogated by anti-CD91 antisera. These studies show that CD91 is essential for re-presentation of gp96-chaperoned peptides by MHC molecules and have an important bearing on the mechanism of immunogenicity of necrotic cells.

heat shock proteins | tumor immunity | α2-macroglobulin | receptor-associated protein | LRP

Heat shock proteins (HSPs) purified from antigen-expressing cells chaperone antigenic peptides generated in that cell (1–4). Immunization with such HSP–peptide complexes purified from tumors or pathogen-infected cells elicits specific immunity directed against the tumor or pathogen, respectively (5–7). The mechanism of immunogenicity of HSP–peptide complexes is increasingly clear. The HSP–peptide complex is targeted to the antigen-presenting cells (APCs) through interaction of HSP with a receptor identified as CD91 (8, 9). In case of the HSP gp96, it appears that the HSP–peptide complex is endocytosed into a compartment positive for Fc receptor and MHC I and negative for Rab5a, CD1, and transferrin (10). The subsequent fate of the HSP molecule itself remains unclear, but the peptide is transported to the cytosol through a mechanism yet to be determined. The peptide is further transported into the endoplasmic reticulum through a transporter-associated with antigen processing-dependent (9, 11) or -independent (see refs. 11 and 12) mechanism. Following a classical pathway within the endoplasmic reticulum, the peptide is charged onto a cognate MHC I molecule, and the MHC I–peptide complex present at the cell surface now stimulates cognate $CD8^+$ T cells. Because the endocytosed HSP–peptide complex is taken up into a compartment that is MHC I^+ (10), the possibility that the gp96-chaperoned peptide is charged onto the MHC I in that compartment, independently of the endoplasmic reticulum, merits consideration.

Among the several HSPs that follow this path, the endoplasmic reticulum-resident HSP gp96 has been the more extensively studied. CD91 was initially identified as the receptor for gp96 through structural means that were functionally corroborated (8). Detergent-solubilized membrane extracts of APCs were applied to gp96-agarose columns, and the bound protein was identified as a fragment of CD91 through MS. Abs to CD91 were shown to inhibit re-presentation of gp96-chaperoned peptides by APCs. Additionally, expression of CD91 was observed to correlate with the ability of an APC to re-present gp96-chaperoned peptides (9). CD91 has been shown to be a receptor for the serum protein α2-macroglobulin (α_2M) (13), and this protein was shown to inhibit re-presentation of gp96-chaperoned peptides. Collectively, these observations led to the suggestion that CD91, a pedigreed receptor for α_2M and a host of other proteins (see ref. 14) is also a receptor for gp96. In independent studies, Habich *et al.* (15) reported that α_2M competes with gp96 for binding macrophage, as does gp96 itself. Banerjee *et al.* (16) showed that Th cells express CD91 on the cell surface and that gp96 binds CD91 on the T helper (Th) cell surface. Such binding is abrogated by anti-CD91 Abs but not control Abs. Additionally, Delneste *et al.* (17) have reported strong inhibition of hsp70 binding to human macrophage by α_2M and a weaker but detectable inhibition of the same reaction on human myeloid dendritic cells (DCs). In contrast to these confirmatory studies, Berwin *et al.* (18) claimed to show CD91-independent re-presentation of gp96-chaperoned peptides; that interpretation derives from lacunae in the experimental design as described in detail in this study.

In this report, we critically examine the genetic and immunological aspects of gp96–CD91 interaction and show, through criteria not previously used, that CD91 plays the dominant role in re-presentation of gp96-chaperoned peptides *in vitro* and *in vivo*.

Materials and Methods

Cell Lines, Mice, and Reagents. B3Z T cell hybridoma was a gift of Nilabh Shastri (University of California, Berkeley) and has been described and characterized (19). The anti-CD91 Ab (rabbit IgG) was made in our laboratory (8). Anti-CD11c Ab-magnetic bead conjugates were purchased from Miltenyi Biotec (Auburn, CA). α_2M was purchased from Sigma and was found to contain the activated/fast form. Full-length recombinant receptor-associated protein (RAP) was a gift from Antigenics (Lexington, MA). Mice were purchased from The Jackson Laboratory and maintained in our vivarium.

Cell-Binding Assays. These assays were carried out as described (20). Cells were analyzed by flow cytometry to obtain the mean fluorescence intensity. Flow cytometry was performed on a FACScan (Becton Dickinson).

Generation of Protein–Peptide Complexes. Gp96 was purified as described (21). α_2M was purchased from Sigma and contained a mixture of activated and nonactivated forms of α_2M as determined by electrophoresis. The AH1–19 (NH_2-RVTYHSPSYVYHQ-

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Abbreviations: α_2M, α2-macroglobulin; APC, antigen-presenting cell; DC, dendritic cell; HSP, heat shock protein; RAP, receptor-associated protein; siRNA, short interfering RNA; VSV, vesicular stomatitis virus.

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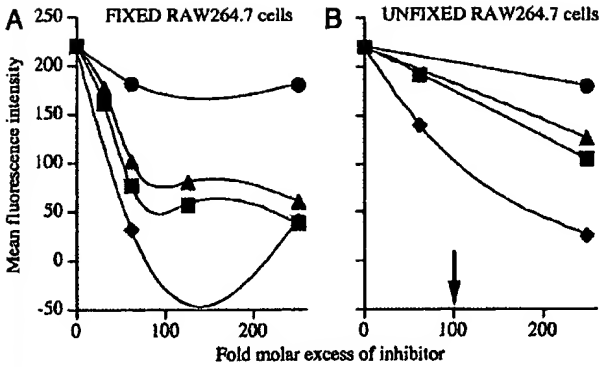

Fig. 1. RAP and α_2M compete with gp96 for binding to CD91$^+$ RAW264.7 cells. RAW264.7 cells were not fixed (*A*) or fixed (*B*) with paraformaldehyde and incubated with gp96-FITC in the presence or absence of increasing molar ratios of unlabeled α_2M, gp96, RAP, or albumin. Unbound protein was removed by extensive washing. The cells were analyzed by flow cytometry. The mean fluorescence intensity was measured and plotted against the fold molar excess of competitor protein mixed with gp96-FITC. The arrow on the *x* axis in *B* indicates the single point at which competition was tested by Berwin *et al.* (18) Three independent experiments were performed. The error bars have been drawn, but are too small to be visible.

FERRAK-COOH), ova20 (NH_2-SGLEQLESIINFEKLTEWTS-COOH), or vesicular stomatitis virus (VSV)20 (NH_2-SLSNLRGYVYQGLKSGNVS-COOH) peptides were synthesized by Genemed Biotechnologies (South San Francisco, CA) to >95% purity. These sequences are derived from gp70, ovalbumin, and VSV nucleoprotein, respectively, with the MHC I-binding epitopes underlined. Protein was heated to 50°C in the presence of 50 M excess of peptide. The protein/peptide mixture was incubated at room temperature for 30 min and then placed on ice. Free, uncomplexed peptides were removed by three centrifugations in Centricon 50 (Millipore), and the depletion was confirmed by electrophoresis. Complexes thus made were used for re-presentation assays.

Re-Presentation Assays *In Vitro*. Re-presentation assays were performed as described (8). Culture supernatants were assayed for IFN-γ by ELISA by using kits from Endogen (Cambridge, MA).

Re-Presentation Assay *In Vivo*. Immunization of gp96–peptide complexes (with or without added Abs) was done in a 100-μl volume, intradermally. This formed a $\leq$5 mm^2 weal. Lymph nodes were harvested after 6 h, crushed, and sorted on an AutoMacs for CD11c$^+$ cells. CD11c$^+$ cells (3×10^4) were incubated with B3Z T cells at a 1:1 ratio for 6 h, fixed, washed, and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (see ref. 19). The number of blue-stained cells was used as a reflection of re-presentation of gp96-chaperoned peptides.

RNA Interference. Short interfering RNA (siRNA) sequences were selected and constructed by Dharmacon (Lafayette, CO). Transfection was conducted as described with few modifications (22). In brief, 3 μl of 20 μM siRNA was incubated with 3 μl of GenePorter transfection reagent (Gene Therapy Systems, San Diego) in a volume of 50 μl of RPMI medium 1640 (serum free) at room temperature for 30 min. This was then added to cells in 300 μl of RPMI medium 1640 plus 10% FCS. Mock control cells received GenePorter alone. An additional 300 μl of complete medium was added 4 h later. Protein expression was then analyzed at various time points. Transfection efficiencies for RAW264.7 were predetermined by using fluorescein-labeled siRNAs.

Tumor Rejection Assays. Tumor rejection assays were carried out as described (2).

Results

RAP and gp96 Compete for Binding to CD91. FITC-gp96 has been shown previously to bind paraformaldehyde-fixed CD91$^+$ RAW264.7 but not CD91$^-$ RAW309Cr.1 cells saturably and competitively (9, 20). Unlabeled gp96 and the previously known CD91 ligand α_2M compete with FITC-gp96 in such assays. These competition studies have now been extended to include the CD91-associated protein known as RAP. RAP is a well known CD91-binding protein that inhibits a number of CD91-mediated activities (23). Gp96-FITC (at a concentration, selected so as to be in the linear range of binding to RAW264.7 cells) was mixed with titrated molar excess amounts of RAP and incubated with paraformaldehyde-fixed RAW264.7 cells. As controls, gp96-FITC was mixed with molar excess of unlabeled gp96, α_2M, or albumin and incubated with fixed cells simultaneously. Cells were washed free of excess protein and analyzed by flow cytometry. The mean fluorescence intensity was measured as an indication of the amount of gp96 bound. As shown in Fig. 1*A*, increasing the amount of competitor protein RAP decreased the amount of gp96-FITC bound to cells. Also, unlabeled gp96 or α_2M competed with labeled gp96 for binding. Albumin, which does not bind to CD91, failed to compete with gp96-FITC for binding. No differences were observed in the competition studies if the competitor was incubated with the fixed cells before addition of gp96-FITC (data not shown).

Although competition of gp96 and α_2M for CD91 have been confirmed by independent studies, Berwin *et al.* (18) failed to observe competition between gp96 and RAP, and gp96 and α_2M for binding to RAW264.7 cells. We have explored and clarified the basis of this apparent discrepancy. First, we have successfully reproduced the experiment as published by Berwin *et al.* (18) (Fig. 1*B*): binding of gp96-FITC to live RAW264.7 cells is competed efficiently by unlabeled gp96 but poorly by α_2M or RAP at the 100-fold molar excess of competitor, the single excess ratio used by Berwin *et al.* (18). Our experiments show 19% inhibition of binding of gp96-FITC by unlabeled α_2M, 21% by unlabeled RAP, and 56% by unlabeled gp96, against a background of 7% inhibition by albumin. These numbers almost exactly reflect the data published by Berwin *et al.* (18). The discrepancy lies in two aspects. In contrast to Berwin *et al.* (18), we have carried out a titrated study at multiple concentrations of competitor, and in such an experiment, significant competition is indeed observed between gp96 and α_2M and RAP at a 200-fold molar excess of α_2M or RAP (Fig. 1*B*). The second source of variation has to do with the fact that our original (8, 20) and present experiments in Fig. 1*A* used paraformaldehyde-fixed cells, whereas Berwin *et al.* (18) and present experiments in Fig. 1*B* use live cells. We have shown (20) and commented upon the fact that CD91 is internalized extremely rapidly (in an order of minutes) upon engagement. Even in experiments done at 4°C, one sees measurable internalization (data not shown). It is for this reason that we do competition studies of binding on paraformaldehyde-fixed cells rather than live cells. The data show that at corresponding competitor concentrations, much clearer inhibition of binding by unlabeled gp96, RAP, or α_2M is seen with fixed cells (Fig. 1*A*) than with live cells (Fig. 1*B*), although significant inhibition can be detected in both conditions; it is notable that the

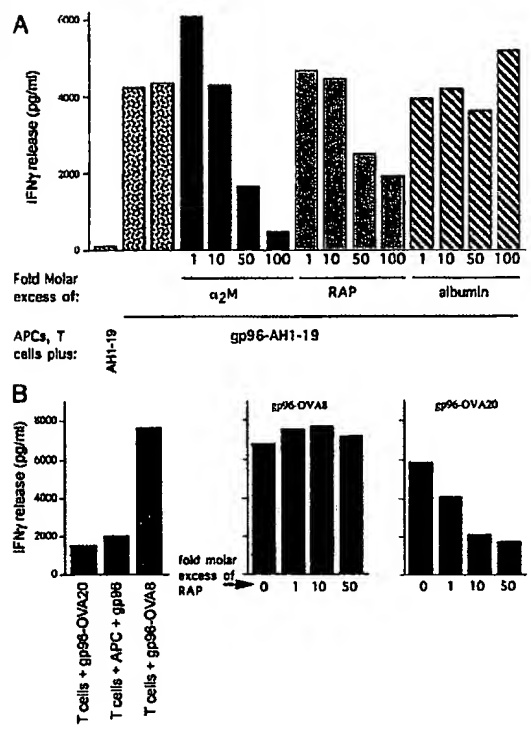

Fig. 2. (*A*) Re-presentation of gp96-chaperoned peptides *in vitro* is inhibited by RAP or α_2M. RAW264.7 cells were used as APCs for re-presentation of gp96–AH1–19 complexes, to AH1-specific T cells. APCs, T cells, and AH1–19 in the absence of gp96 showed no T cell stimulation (first bar). Re-presentation requires APCs, T cells, and gp96 chaperoning AH1–19. Re-presentation is inhibited by increasing quantities of RAP or α_2M but not albumin mixed with the gp96–AH1–19 complexes and incubated with RAW264.7 and T cells. Release of IFN-γ was monitored as a marker of T cell stimulation. The IFN-γ contents of each well are indicated. Each well was tested in duplicate. (*B*) Re-presentation is distinct from surface charging. Re-presentation of ova8 peptide by APCs to T cells was performed with gp96 complexed to ova8 or ova20 peptide. The gp96–peptide complexes were washed with a centricon to remove excess uncomplexed peptides. Re-presentation of peptides was inhibited with RAP at the doses indicated. Control groups (*Left*) include T cells incubated with gp96 complexed to ova8 or ova20 in the absence of APCs, T cells plus APCS and gp96 (uncomplexed), and T cells plus APCs. Three independent experiments were performed. Error bars have been indicated, but are too small to be visible.

extent of inhibition seen with unlabeled albumin is constant under the two conditions.

RAP Inhibits Re-Presentation of gp96-Chaperoned Peptides *in Vitro*. The ability of RAP to inhibit re-presentation of gp96-chaperoned peptides by RAW264.7 cells was tested. Because re-presentation requires internal processing (9), these experiments were carried out with live cells at 37°C. Gp96 was complexed to AH1–19, a 19-mer peptide containing the underlined AH1 sequence (NH_2-RVTYHSPSYVYHQFERRAK-COOH), which is an L^d-restricted epitope (8). Gp96-AH1–19 was incubated with RAW264.7 and a $CD8^+$ T cell clone that recognizes the L^d/AH1 in a re-presentation assay, under conditions described in *Materials and Methods*. Stimulation of the $CD8^+$ clone requires uptake and processing of gp96-chaperoned AH1–19 by the RAW264.7 cells; stimulation is read as secretion of IFN-γ by the T cells in a 20-h assay. In separate wells, RAP, α_2M, or albumin were added to the assay in molar excess as indicated in Fig. 2*A*. Gp96-chaperoned peptides were re-presented efficiently in the absence of a competitor, and the degree of re-presentation was diminished significantly and titratably when either α_2M or RAP was added in increasing amounts. Albumin did not compete with gp96 at any concentration tested. Greater inhibition was observed with α_2M than with RAP. The reason for this observation is not entirely clear. RAP is known to bind to ligand-binding clusters II–IV of CD91, whereas α_2M is known to bind to clusters II and IV (14, 23). The relative affinities of interactions of RAP and α_2M to CD91 have been reported to be similar in fluid phase studies *in vitro*. These values may or may not be reflective of affinities in a live cell-based assay such as the re-presentation assay used here. Furthermore, the differences between α_2M and RAP in inhibiting re-presentation of gp96-chaperoned peptides may derive from differential allosteric conformational changes introduced into the large CD91 molecule by binding of gp96 to it.

Berwin *et al.* (18) have reported that re-presentation of gp96-chaperoned peptides is not inhibited by RAP or α_2M. This result derives from an assay that does not measure re-presentation but surface charging of MHC I molecules. When the re-presentation assay was first described in 1995 (24), the authors took pains to distinguish re-presentation of gp96-chaperoned peptides from direct charging of the peptides onto the surface MHC I molecules of APCs by the use of extended precursors of antigenic epitopes, which cannot directly charge surface MHC I molecules. As an example, we show in Fig. 2*B* that, although the ovalbumin-derived SIINFEKL peptide can charge an APC directly (*Center*), its 20-mer-extended version NH_2-SGLEQLESIINFEKLTEWTS-COOH cannot do so (*Right*) and therefore is the appropriate peptide for a re-presentation assay. This precaution of seminal significance was not taken by Berwin *et al.* (18). The ovalbumin-derived SIINFEKL peptide used in that study was complexed to gp96 as an 8-mer and not as an extended variant. We have reproduced the experiment of Berwin *et al.* (18) and, indeed, observe that, as reported there, re-presentation of gp96-chaperoned SIINFEKL is not inhibited by RAP (Fig. 2*B Center*). In contrast, re-presentation of gp96-chaperoned NH_2-SGLEQLESIINFEKLTEWTS-COOH is inhibited by excess RAP titratably and completely, to background levels (Fig. 2*B Right*).

CD91-Dependent Re-Presentation of gp96-Chaperoned Peptides *in Vivo*. On the shaved mid-ventral aspect of each mouse, 1 μg of g96–ova20 complex was introduced intradermally. The axillary and inguinal draining lymph nodes were harvested 6 h after immunization and $CD11c^+$ cells isolated by magnetic sorting to 92% purity. These $CD11c^+$ cells were used for stimulation of B3Z cells, a T cell hybridoma that synthesizes β-galactosidase when its T cell receptor engages the K^b/SIINFEKL complex (19). Synthesis of β-galactosidase can be monitored by development of the blue color (due to hydrolysis of 5-bromo-3-indoyl-β-D-galactopyranoside) upon treatment of B3Z cells with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the number of cells in which the blue color develops is an indication of stimulation of B3Z, which in turn is an indication of the extent of re-presentation of gp96-chaperoned peptides by the MHC I of the DCs. Fig. 3*A* shows B3Z cells were stimulated when gp96–ova20 but not gp96–VSV20 was injected, demonstrating the specificity of re-presentation *in vivo*. The dependence of this system on gp96 was demonstrated by the inability of albumin–ova20 complexes, ova20 alone, or PBS to show a positive signal. To test the involvement of CD91 in such re-presentation *in vivo*, mice were immunized with gp96–ova20 complexes together with the anti-CD91 Ab (8) or a control Ab. In other groups, α_2M or albumin was added to the immunogen. $CD11c^+$ cells obtained from all groups of mice were tested for B3Z T cell stimulatory capacity. Fig. 3*B* shows that $CD11c^+$ cells from mice immunized with gp96–ova20 complexes in the presence of the anti-CD91 Ab failed to stimulate B3Z T cells; an inhibition of >70% was observed compared to immunization with gp96-ova20 in PBS. No significant inhibition

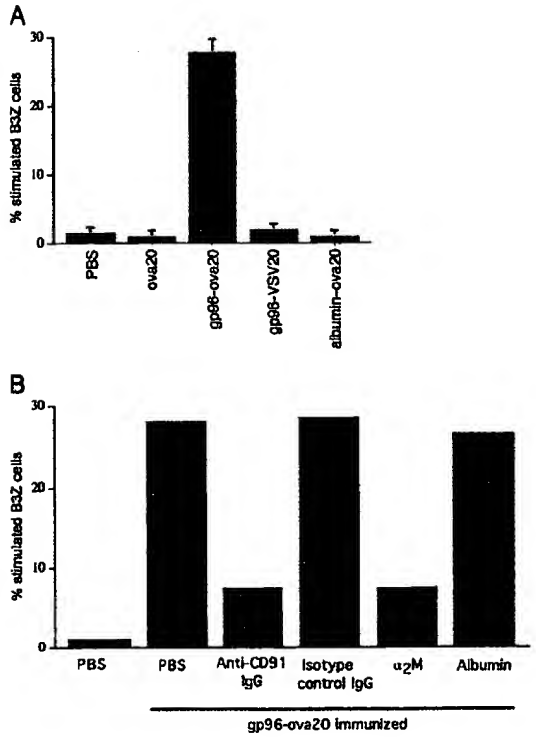

Fig. 3. Re-presentation *in vivo* is CD91 dependent. (*A*) Mice were immunized with gp96–ova20 or gp96–VSV20 complexes. After 6 h, $CD11c^+$ cells were harvested from the lymph nodes and used for stimulation of B3Z cells as described in *Materials and Methods*. Percentage of stimulated B3Z cells is plotted. As an additional control for specificity, mice were immunized with albumin–ova20, ova20 alone, or PBS, and lymph nodes were similarly analyzed. Lymph node cells from mice immunized with gp96–ova20, but not mice immunized with albumin–ova20 or ova20 alone stimulated B3Z cells. (*B*) Mice were immunized with gp96–ova20 complexes alone or mixed with anti-CD91 or control antisera, or with α_2M or albumin. Lymph node cells were isolated and used to stimulate B3Z cells. The percentage of stimulated B3Z cells is plotted. The average percentage for two mice is shown in each group. These experiments have been carried out in triplicate and the error bars are shown. In some groups, error bars are too small to be visible.

(<1%) was seen with control Ab. A 10-fold molar excess of α_2M also inhibited re-presentation of gp96-chaperoned peptides as efficiently as the anti-CD91 Ab, whereas a 100-fold molar excess of albumin did not do so.

Genetic Evidence for the Essential Role of CD91 in Re-Presentation. CD91 is an essential molecule and CD91-knockout mice are embryonic lethal. In light of this limitation, CD91 siRNA was used to create APCs whose CD91 expression has been extinguished. Re-presentation-competent RAW264.7 cells were transfected with siRNAs for CD91 or actin (as control), and expression of CD91 was monitored. Transfection of RAW264.7 cells with CD91 siRNA led to detectable dimunition of expression of CD91 within 12 h of transfection. Virtually no CD91 was detectable at 24 and 48 h after transfection. Recovery of CD91 expression began to be seen at 60 h after transfection (Fig. 4*A*). There was no dimunition of actin expression in cells transfected with CD91 siRNA, nor was there any alteration in the overall protein profile in CD91 siRNA-transfected cells. Conversely, transfection of cells with actin siRNA did not affect CD91 expression, whereas it did extinguish expression of actin. CD91 siRNA- and actin siRNA-transfected RAW264.7 cells were tested for their ability to bind CD91 ligands FITC–α_2M or FITC–gp96 and phycoerythrin (PE)-anti-CD11b Ab as a positive control (Fig. 4*B*). CD91 siRNA-transfected cells showed dramatic extinction of the ability to bind FITC–gp96 and FITC–α_2M; the ability of these cells to bind anti-CD11b Ab remained unaffected. Actin siRNA-transfected cells showed no dimunition of binding to any ligand tested.

CD91 siRNA-transfected cells were tested functionally for their ability to re-present gp96-chaperoned and α_2M-chaperoned antigenic peptides by using the L^d-restricted AH1 system described and used for Fig. 2*A*. It was observed (Fig. 4*C*) that transfection with CD91 siRNA completely abolished the ability of RAW264.7 cells to re-present peptides chaperoned by gp96 or α_2M, when RAW264.7 cells were tested 24–48 h after they were transfected. Interestingly, when RAW264.7 cells transfected 60–80 h earlier were tested, they were fully able to re-present peptides chaperoned by gp96 or α_2M. It may be recalled from Fig. 4*A* that RAW264.7 cells lose CD91 expression 24–48 h after transfection with CD91 siRNA but begin to recover the expression starting at 60 h. Actin siRNA-transfected APCs remained fully competent to re-present.

Abrogation of Tumor Immunity by Inhibiting gp96–CD91 Interactions. Because re-presentation is necessary for tumor immunity elicited by tumor-derived endogenously generated gp96–peptide complexes, we examined the effect of inhibiting gp96–CD91 interaction on tumor immunity. Naive 6- to 8-wk-old BALB/c mice were immunized twice at weekly intervals with gp96–peptide complexes purified from the methylcholanthrene-induced sarcoma, Meth A (or as a negative control, gp96–peptide complexes purified from normal BALB/c mouse liver), as described in ref. 21 and in *Materials and Methods*. Mice were challenged with 100,000 Meth A cells intradermally, and kinetics of tumor growth was monitored (Fig. 5). This prototypical experiment has been published extensively and shows that mice immunized with Meth A–gp96 are tumor-immune, whereas those immunized with liver–gp96 are not. To this experiment, we have added the anti-CD91 or control Ab, along with Meth A–gp96. The results (Fig. 5) show that Meth A–gp96 is unable to elicit protective tumor immunity in presence of anti-CD91 Ab ($P < 0.0001$ compared to control Ab on day 17).

Discussion

Identification of CD91 as a gp96 receptor was based on structural studies followed by functional inhibition of gp96 activity by anti-CD91 Ab and by α_2M, a major independent ligand for CD91. The present experiments complement and extend those studies, and by use of genetic methods, now prove that CD91 is essential for re-presentation of gp96-chaperoned peptides.

The data by using CD91 siRNA demonstrate that as APC lose expression of CD91, their re-presenting ability undergoes a corresponding and dramatic decline. Furthermore, as the cells recover from extinction of CD91 expression, they regain the ability to re-present peptides. The ability of cells to bind α_2M, a previously known CD91 ligand, or HSP gp96, and their ability to process peptides chaperoned by α_2M undergo identical variations. Additionally, the results show inhibition of several gp96-mediated effects (binding to an APC, re-presentation *in vitro* and *in vivo*) by another well known ligand of CD91, the RAP. The literature on gp96–CD91 interaction contains some contradictory results (8, 9, 18), and these discrepancies are explained and resolved. The first evidence of a role for CD91 in tumor rejection *in vivo* with endogenously purified gp96 is presented. Administration of anti-CD91 Ab but not control rabbit Ig inhibits gp96-elicited protective tumor immunity completely. This observation provides a methodological window into exploration *in vivo* of gp96–CD91 interactions.

In addition, we report here the development of an unique re-presentation assay that can measure re-presentation *in vivo* and

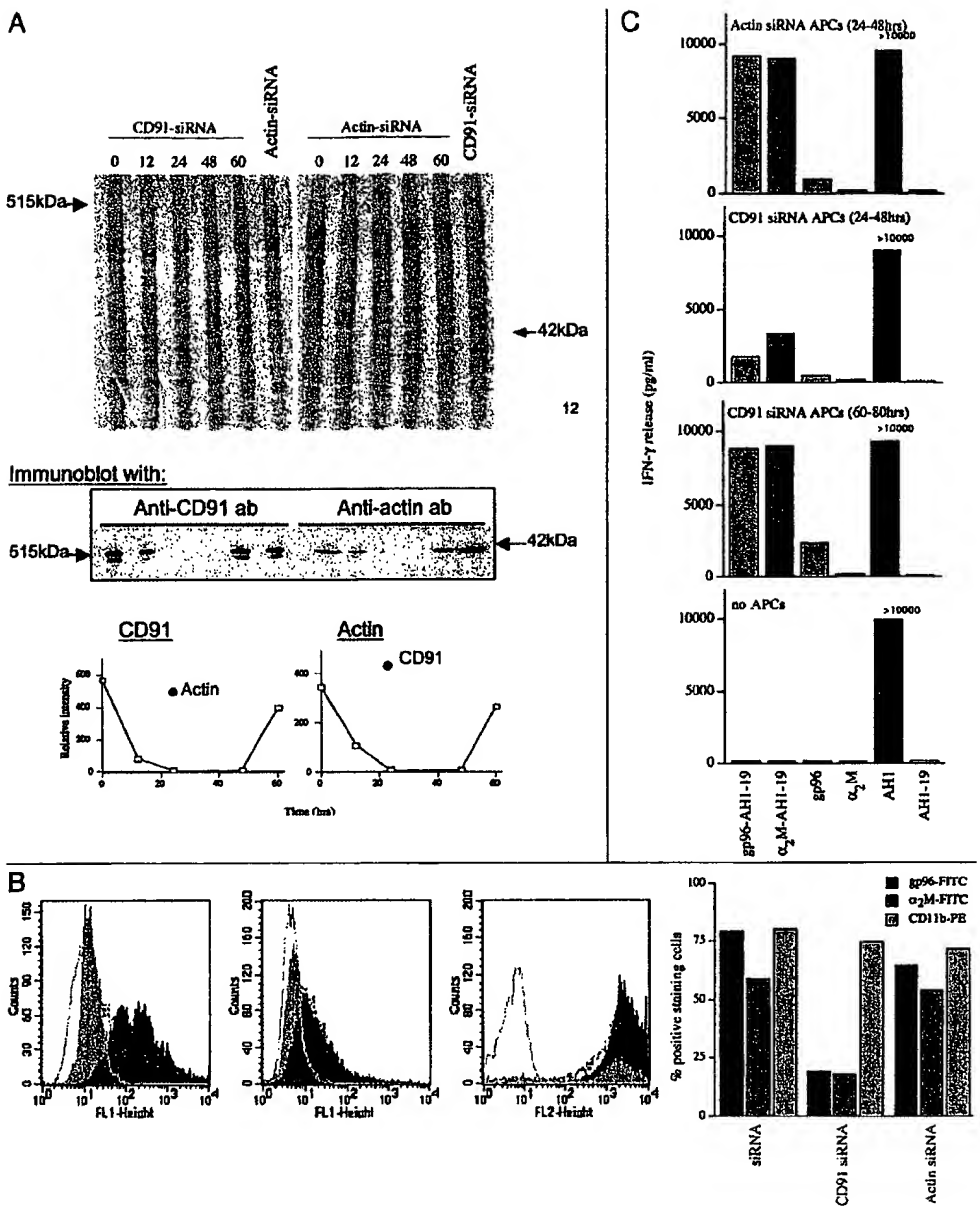

Fig. 4. APCs deficient in CD91 fail to bind gp96 and to re-present gp96-chaperoned peptides. (*A*) RAW264.7 cells treated with CD91–siRNA lose expression of CD91. Cells were incubated with the indicated siRNA and harvested at various time intervals over a 60-h period. Lysates were resolved by PAGE (*Top*) and immunoblotted (*Middle*) for CD91 as described in *Materials and Methods*. The relative intensities of the immunoblot bands were scanned and plotted against time (*Bottom*). The PAGE analysis shows that transfection with CD91 or actin siRNA does not lead to a discernible alteration in the overall protein pattern of the transfected cells. Immunoblots and graphs show that siRNA-transfected cells begin to lose expression of CD91 by 12 h and that the expression becomes undetectable by 24 h, stays undetectable for another 24 h, and recovers by 60 h. The experiments were also done with actin–siRNA as a specificity control with similar results. Actin–siRNA did not affect CD91 expression and vice versa. (*B*) Loss of CD91 expression correlates with decreased α_2M and gp96 binding. FITC-labeled gp96 (*Left*), α_2M (*Middle*), or anti-CD11b Ab (as a control, *Right*) were used as ligands in binding studies with untreated RAW264.7 cells (dark shaded histograms) or the same cells treated with CD91–siRNA (light shaded histograms) or actin–siRNA (dotted lines) for 24 h. Decreased binding of gp96 and α_2M to CD91–siRNA-treated RAW264.7 cells, compared to actin–siRNA or untreated cells, was observed. No dimunition of binding of anti-CD11b Ab was observed on cells transfected with CD91 or actin siRNAs. The number of cells staining positive in each group from these histograms is plotted in the bar graph at far right. (*C*) Loss of CD91 expression abrogates re-presentation of gp96- or α_2M-chaperoned peptides. RAW264.7 cells, untreated or treated with CD91–siRNA or actin–siRNA, were used as the APCs in re-presentation assays with gp96–AH1–19 or α_2M–AH1–19 complexes and a T cell line specific for AH1 as in Fig. 2. Re-presentation of AH1 was abrogated when APCs were treated with CD91–siRNA compared to untreated or actin–siRNA APCs within 24–48 h of transfection. The loss of re-presentation ability recovers at 60 h, correlating with the recovery of CD91 expression (see *A*). APCs are necessary for re-presentation because there is no IFN-γ release in their absence. Two independent experiments were performed. Error bars have been indicated, but are too small to be visible in *B* and *C*.

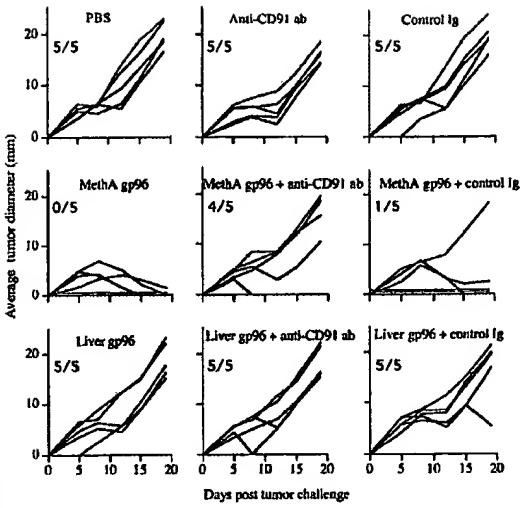

Fig. 5. Anti-CD91 Ab inhibits rejection of Meth A fibrosarcoma elicited by immunization with Meth A-derived gp96. Mice were immunized twice, 1 week apart, with tumor- or liver-derived gp96 alone, or in the presence of anti-CD91 or control Abs. In case of mice that received Abs, Ab was also administered the day of and 2 days after each immunization with gp96. Mice were challenged with tumor cells 1 week after the last gp96 immunization, and tumor size was monitored. Kinetics of tumor growth in individual mice is shown. Mice immunized with Meth A-derived gp96 alone or mixed with control antisera rejected the tumor challenge (zero of five or one of five mice, respectively, grew tumors), but mice immunized with Meth A-derived gp96 mixed with anti-CD91 Ab failed to reject their tumors (four of five grew tumors). ($P < 0.0001$ for Meth A gp96 + anti-CD91 Ab vs. Meth A gp96 + control Ig on day 17). One of two complete, independent experiments is shown.

with specificity and that complements the re-presentation assay in current use that is exclusively *in vitro* (8). This assay requires much smaller quantities of immunogen (1 μg per data point) than the current assay *in vitro* (several tens of micrograms per data point) and provides an opportunity to test the role of other immunological compartments *in vivo* through the use of knockout mice and neutralizing Abs.

CD91 was identified as a receptor for gp96 followed soon after by the observation that it also acts as a receptor for other re-presenting HSPs such as hsp70, hsp90, and calreticulin (9). Subsequent studies have provided independent evidence for CD91–calreticulin interactions (25, 26). The receptivity of CD91 for various HSPs that do not share any sequence or other structural homologies would have been surprising were it not for the fact that this primitive molecule (conserved at least as far back as *Caenorhabditis elegans*) has at least 25 other known ligands that also do not share obvious structural homologies among each other. Part of the promiscuity of CD91 derives from its rich multidomain secondary and tertiary structures. Nonetheless, even a single domain of CD91 is able to interact with multiple ligands (e.g., domain II and its ligands *Pseudomonas* exotoxin A, α_2M, and RAP) that too share little apparent structural similarity, thus confounding the problem. The early structural studies suggested that gp96 interacts with CD91 at its N-terminal 80-kDa end that is not known to bind other ligands. Efforts to quantify the gp96–p80 interaction as well as to define the gp96 domain that interacts with CD91 should aid in further understanding of HSP–CD91 interactions.

The studies on HSP–CD91 interactions thus far have been restricted to mice, but parenthetical evidence of their existence and its significance in the human immune system has begun to appear. Thus, Delneste *et al.* (17) have reported strong inhibition of hsp70 binding to human macrophage by α_2M and a weaker but detectable inhibition of the same reaction on human myeloid DCs. Delneste *et al.* (17) have identified Lox-1, a member of the same scavenger superfamily as CD91, as an additional receptor for endocytic uptake of hsp70 and chaperoned peptides by human DCs. The proposed role of Lox-1 appears to be unique for the human system because inhibition of CD91 is able to account fully for the same activity in the murine system (9). Curry *et al.* (27) have observed the ability of HSPs to cause maturation of blood-derived monocytes and expression of CD91 on dermal DCs. In a collaborative small study of 18 patients, Stebbing *et al.* (28) have reported that monocytes of therapy-naive long-term nonprogressors with HIV infection express higher (and statistically significant, $P < 0.01$) levels of CD91 than progressors. The difference in CD91 expression is the only variable among many that cosegregates with long-term nonprogression of HIV in these patients. The authors suggest that a higher expression of CD91 may confer a proportionately higher capacity of APCs of these individuals to cross-present viral antigens to the patients' T cells. In another study in patients with Kaposi's sarcoma, Stebbing *et al.* (29) observed that sequestration of HSPs in lysates of virus-infected cells by the use of HSP-selective antibiotics or the blockade of CD91 by anti-CD91 Ab lead to a significant inhibition of T cell activation *in vitro*. Consistent with the murine studies, these studies are suggestive of a role of HSP–CD91 interaction in the human immune system and require further interrogation with more precise experimental strategies.

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